Ultrastructural Changes in Muscular Dystrophy

I. Cardiac Tissue of Piglets Deprived of Vitamin E and Selenium

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The ultrastructure of cardiac tissue from neonate, 3-, 6-, 8-, and 12-week-old piglets, born of vitamin E- and selenium-deprived sows was studied. A progression of lesions occurred in nonmuscular components of this tissue; the first lesion appeared in connective tissue elements. Fibroblasts and the extracellular compartment appeared most severely altered in the neonate, and progressive vascular damage was very evident from 3 to 12 weeks of age. Similarly, neuronal elements appear altered at 3 weeks and were almost nonevident in areas showing marked lesions at 8 and 12 weeks. Fairly extensive alterations were evident in all of these elements before any marked changes become evident in the muscle. The relevance of these observations is discussed in relation to the etiology of the disease (Am J Pathol 68:479–492, 1972).

NUMEROUS REPORTS ARE AVAILABLE on the production of cardiac muscular dystrophy in swine after nutritional deficiencies in vitamin E and selenium,[1–4] and the condition is commonly referred to as *mulberry heart* disease. Although numerous reports of histologic observations of this disorder are available, nothing has been published on ultrastructural changes in affected tissues.

Changes in the cardiac tissue of piglets born of vitamin E- and selenium-deficient sows were studied. Gross lesions were examined ultrastructurally but due to the complexities of observed cell alterations, an experiment was designed so that cardiac tissue from day 0 postnatally to 12 weeks of age could be collected and the sequence of changes established. This paper presents the ultrastructural changes observed during this investigation; primary consideration will be given to nonmuscular elements, since those appeared to be the first affected by the deficiency.

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Materials and Methods

All animals used in this study were piglets born to vitamin E- and selenium-deficient sows that had been maintained throughout gestation and lactation on a modified version of the Brown *et al*, diet [5]—vitamin E was not included and the soybean oil meal used was made from soybeans grown in selenium-poor soil. Analyses of the diet showed that it contained less than 0.05 ppm selenium. It was used as creep feed for the young from age 10 days to 3 weeks, after which time they were weaned and maintained solely on the diet. After weaning, half of the piglets received selenium as injected sodium selenate, 0.5 mg in sterile saline weekly, and vitamin E, 1000 IU in ethanol was given intramuscularly, weekly. Selected experimental animals were sacrificed at birth and at 3, 6, 8 and 12 weeks of age.

All cardiac tissues, normal or experimental, obtained for this study were collected from the right and left ventricles and the right atrium. They were immediately placed in appropriate fixative and cut into 0.75 mm cubes. Glutaraldehyde, in concentrations of 1.5, 2, 3 and 5% in 0.1, 0.15 and 0.2 M Sorensen's phosphate buffer at pH 7.2 to 7.3, was used as fixative. All samples were postfixed in 1% osmium tetroxide in phosphate buffer with 0.5% sucrose, pH 7.2 to 7.3. Other samples of all tissues were fixed only in 1% osmium tetroxide in phosphate buffer with 0.5% sucrose.[6] All fixations were performed at 4 C for 2 hours; subsequent to fixation, all tissues were dehydrated in acetone, (Baker analytical), and embedded in Epon.[7] Thick (1 μ) sections of all blocks were cut, stained with methylene blue [8] and examined by light microscopy for lesions or to confirm the presence of desired structures so that the proper areas of tissue would be thin-sectioned. All subsequent thin sections were cut on either a Porter Blum Ultramicrotome or a Reichert OMU2, using glass knives. The sections were placed on unsupported grids, stained with uranyl acetate and lead and examined in a Philips electron microscope, model 200.

Results

Electron microscopic examination revealed that normal cardiac tissue contained typical cardiac muscle cells with prominent intercalated discs and large quantities of mitochondria. The sarcoplasm contained ribosomes, the sarcoplasmic reticulum appeared normal, and the sarcomeres appeared to be in a state of contraction (Figure 1). In addition to mature contractile elements, myoblasts were seen on numerous occasions in specimens from animals up to 6 weeks of age; these are illustrated in Figure 2.

The noncontractile components of normal tissue include fibroblasts and small vessels (capillaries, arterioles and venules), as well as larger vessels, nonmyelinated neurons and extracellular collagen. In all ages examined, fibroblasts showed prominent dilations of ergastroplasmic cisternae containing an electron-dense, flocculent material (Figures 3, 4). The cytoplasm of these cells contained mitochondria, ribosomes and an occasional lipid-like droplet (Figure 4). The neurons contained all classic organelles: mictochondria, microtubules and neurosecretory granules. Occasional fibers con-

tained electron-dense, lipid-like inclusions (Figure 5). The extracellular compartment contained relatively large quantities of collagen fibrils and, in some areas, an amorphous flocculent material was visible (Figures 3, 4, 5); the basal lamina was thin and distinct around muscles, vessels (Figure 3) and nerves (Figure 5).

Initial examination of those areas of cardiac tissue from deficient piglets, which visually appeared pathologic, revealed a complex array of degenerate muscle, nerves, vessels and fibroblasts (Figure 6). It was thus decided to study earlier stages of this disease in an effort to ascertain which tissue components were the first to show ultrastructural changes. At 3 to 6 weeks of age, the only major change visible in cardiac muscle cells was the appearance of lipid and evidence of mild mitochondrial degeneration (Figure 7). The most pronounced changes occurred in vessels and in the extracellular compartment. The predominant capillary changes involved varying degrees of endothelial degeneration, ranging from images showing slight loss of cytoplasmic density to excessive ballooning, with complete lack of all cytoplasmic organelles and organization (Figures 7–10). In addition to these alterations of the endothelium, electron-dense, lipid-like granules could be seen in vascular elements, degenerate neurons (Figures 7, 12) and fibroblasts; the latter appeared ruptured and degenerated (Figure 7). Numerous instances of major changes in neurons were seen wherein the axons appeared dilated and contained no discernable organelles (Figure 11); Schwann cells contained dilated endoplasmic reticulum as well as electron-dense inclusions (Figure 12).

The extracellular compartment of tissues at 6 and 8 weeks of age did not contain significant quantities of mature collagen bundles but did appear to contain a flocculent precipitate (Figures 6, 9, 11), 12). In addition, the basal lamina appeared thicker and more fibrous in nature in vessels which were most degenerate (Figure 10) and discontinuous in vessels with minimum alterations (Figures 8, 9). Thus, even at 3 weeks of age, significant alterations in most nonmuscular elements can be readily ascertained at the ultrastructural level. It should be emphasized that the above alterations in structure were not found throughout the tissue blocks examined, but only in restricted areas; they were most prevalent in the left ventricle.

It was then decided to examine tissues of newborn piglets of vitamin E- and selenium-deprived sows in the hope that perhaps only one of the above lesions would be observed. Light microscopic examination of these neonatal cardiac tissues revealed no apparent

differences between them. Ultrastructural studies, however, revealed alterations in the capillaries and fibroblasts, as well as in connective tissues. The severity of lesions in the capillary endothelium progressed from normal endothelium to the mildest lesion at 6 weeks (compare Figures 13 and 7). No gross ballooning was seen at this stage with any fixation technic used. The most pronounced alterations were found in the fibroblasts, most of which appeared ruptured, with only scanty profiles of dilated endoplasmic reticulum (Figure 14). Normal fibroblasts were seen in certain areas and were similar to those at 3 to 6 weeks. In addition to these cellular changes, the extracellular compartment was almost devoid of the collagen bundles prevalent in controls. This compartment, however, contained large quantities of very fine filments which did not show axial periodicity, although they are found in close association with fibrils which do show the classic periodicity of collagen (Figure 15). These filaments were occasionally seen in controls but appeared to constitute the majority of extracellular filaments in the deficient piglet.

Discussion

In view of the above observations, it seems that there are two very early lesions in cardiac tissue of piglets from vitamin E- and selenium-deprived sows. These appear in connective tissue and capillaries and precede any apparent structural changes in muscle cells proper. Because the fibroblasts appear more degenerate than do the capillaries in newborns, it is felt that the deficiency has its most pronounced affect on this cell population and that capillary changes result from alterations in the connective tissue component of this organ.

The possibility that we are dealing with a connective tissue alteration is supported by the observations of King and Maplesdon [9] and Bourne and Golarz.[10] These authors suggest that changes occur in the polysaccharides of tissue; similar observations have been reported in muscle tissue of chickens suffering from genetic muscular dystrophy.[11] Brown *et al* [12] reported that avitaminosis E in rats increased the amounts of soluble collagen in the skin, decreased the rates of fibril formation *in vitro* and decreased the stability of fibrils *in vitro*. These data suggested that vitamin E was involved in normal fibril formation. Further studies [13–15] revealed that, although serum ascorbate remains relatively normal in these piglets, the ascorbate levels of cardiac tissue shows a continuous decline from birth. The role of this molecule in normal connective tissue metabolism is well known,[16] and its apparent loss from muscle would imply an altera-

tion in connective tissue maturation, particularly fibrillogenesis. Our observations suggest an alteration in collagen fibril formation at early stages of the deficiency which could result from a decrease in available ascorbate. Indeed, Supplee [17] has shown that the feather abnormalities associated with nutritional muscular dystrophy in the chicken are partly protected by ascorbate.

It is apparent from our observations that myogenesis is still active up to 6 weeks postnatally and, therefore, differentiation of this organ is far from complete in the neonate. Thus full consideration must be given to developmental parameters in evaluating the effect of this deficiency on cardiac tissue. Recent evidence by Hauschka and Konigsberg [18] and Hauschka [19] has shown the influence of collagen on the *in vitro* differentiation of muscle. Their data show that the differentiation of cultured myoblasts is markedly enhanced if the cells are grown on a collagen substrate. The data of James [20] and Lieberman [21] also suggest the prominent role collagen development may have on the normal differentiation of the bundle of His and Purkinje fibers in the heart. There is thus ample evidence available in the literature to support the possibility that the primary site of action of the deficiency is the connective tissue compartment of the organ concerned, as suggested by our observations. If such were the case, capillary and nonmyelinated nerve damage would be secondary. Also relevant is the fact that in our experiments lesions appear first in the left ventricle, which postnatally is known to undergo a major burst of growth.[22] This rapid growth and its attendant activity would require effective tissue support system to maintain maximum cardiac output. In this regard, the effects of the deficiency are most pronounced on those muscle populations undergoing rapid growth with superimposed functional demands; this has been confirmed in studies on lamb and chickens.[23] Further studies are presently underway in an effort to elucidate early changes in tissues from other animals deprived of vitamin E and selenium.

References

1. Carpenter LE, Lundberg WO: Effect of tocopherols on viability of pigs in relation to baby pig disease. Ann NY Acad Sci 52:269–275, 1949
2. Lanneck K, Lindberg P, Nilsson G, Nostrom G, Orstadius K: Production of vitamin E deficiency and muscular dystrophy in pigs. Res Vet Sci 2:67–72, 1961
3. Ewan RC, Wastell NE, Bicknell EJ, Speer VC: Performance and deficiency symptoms of young pigs fed diets low in vitamin E and selenium. J Animal Sci 29:912–915, 1969

4. Sharp BA: Experimental induction of vitamin E and selenium responsive lesions in growing swine. MSc Thesis, University of Guelph, Guelph, Ontario, Canada, 1970

5. Brown RG, Sharma VD, Young LG: Ascrobic acid metabolism in swine. Interrelationships between the level of energy intake and serum ascorbate levels. Canad J Animal Sci 50:605–609, 1970

6. Millonig G: Advantages of a phosphate buffer for O_sO_4 solutions in fixation. J Appl Physiol 32:1637, 1961

7. Luft JH: Improvements in epoxy resin embedding techniques. Biochim Biophys Acta 9:409–414, 1961

8. Jeon KW: Simple method for staining and preserving epoxy resin embedded animal tissue sections for light microscopy. Life Sci 4:1839–1841, 1965

9. King JM, Maplesden DC: Nutritional muscular dystrophy in calves. III. Purkinje fiber degeneration in calves with experimentally produced nutritional muscular dystrophy. Canad Vet J 1:421–426, 1960

10. Bourne GH, Golarz MN: Histochemical evidence for a possible biochemical lesion in muscular dystrophy. J Histochem Cytochem 11:286–288, 1963

11. Cosmos E: Ontogeny of red and white muscles: the enzymatic profile and lipid distribution of immature and mature muscles of normal and dystrophic chickens, Physiology and Biochemistry of Muscle as a Food. Edited by EJ Briskey, RG Cassens, BB Marsh. University of Wisconsin Press, 1970, pp 193–207

12. Brown RG, Button GH, Smith JT: Effect of vitamin E deficiency on collagen metabolism in the rat's skin. J Nutr 91:99–106, 1967

13. Brown RG, Partridge IG, Sharp BA: Ascorbate in hepatosis dietetica and mulberry heart disease. J Animal Sci 33:1147 1971 (abstr)

14. Brown RG, Sharp BA, Young LG, Van Dreumel A: Serum ascorbate levels in swine with cardiac muscular dystrophy and hepatosis dietetica. Canad J Animal Sci 51:537–539, 1971

15. Brown RG, Sweeny PR: Ascorbate metabolism in swine changes in tissue ascorbate levels in vitamin E and selenium deficiency. Unpublished data

16. Robertson Wm Van B: The biochemical role of ascorbic acid in connective tissue. Ann NY Acad Sci 92:159–167, 1961

17. Supplee WC: Feather abnormality in poults fed a diet deficient in vitamin E and selenium. Poultry Sci 45:852–854, 1966

18. Hauschka SD, Konigsberg IR: The influence of collagen on the development of muscle colonies. Proc Nat Acad Sci 55:119–126, 1966

19. Hauschka SD: Clonal aspects of muscle development and the stability of the differentiated state, The Stability of the Differentiated State. Edited by H Ursprung. New York, Springer Verlag, Inc, 1968, pp 37–57

20. James RN: Cardiac conduction system: fetal and postnatal development. Am J Cardiol 25:213–226, 1970

21. Lieberman M: Physiologic development of impulse conduction in embryonic cardiac tissue. Am J Cardiol 25:279–284, 1970

22. Goss RJ: Adaptive growth of the heart, Cardiac Hypertrophy. New York Academic Press, Inc, 1971, pp 1–10

23. Sweeny PR, Buchanan-Smith J, deMille F, Pettit JR: Ultrastructural changes in muscular dystrophy. II. Comparative studies on lamb and chicken. Am J Pathol 68:493–510, 1972

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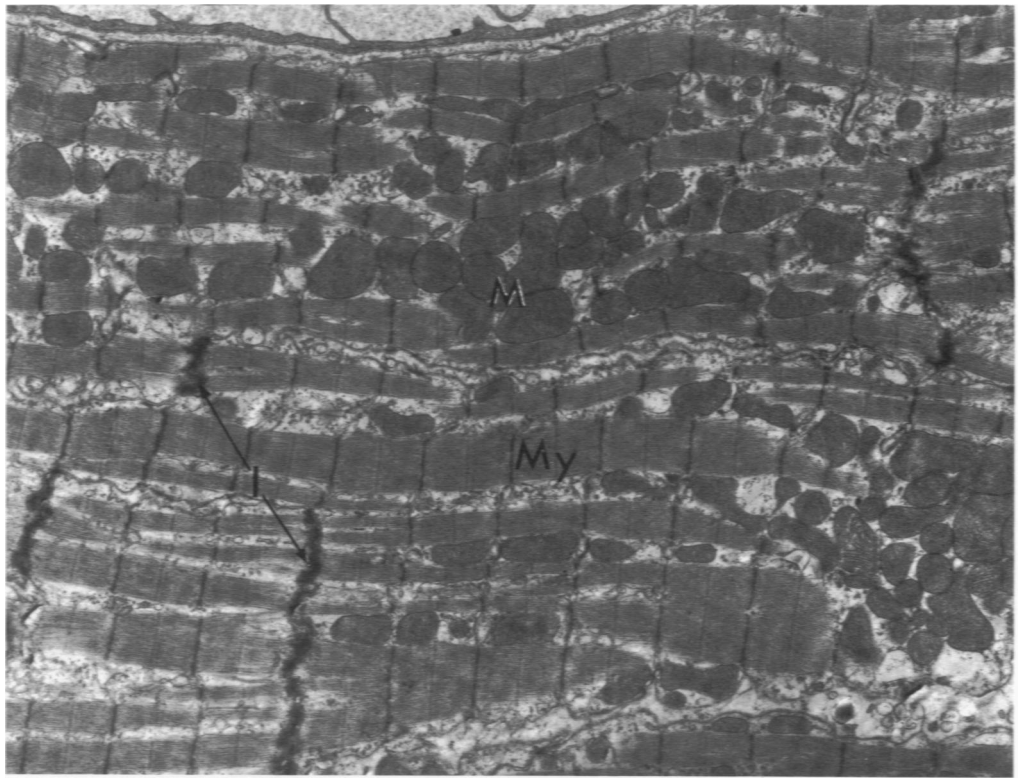
[Illustrations follow]

Fig 1—Electron micrograph of normal cardiac muscle cell showing myofilaments *(My)*, intercalated discs *(I)* and mitochondria *(M)*. Endothelium of a large vein can be seen at the top of the figure (Six weeks, osmium tetroxide uranyl acetate and lead stain, × 6300).

Fig 2—Electron micrograph of two myoblasts from normal tissue showing the immature myofilaments *(My)*, scattered mitochondria *(M)*, sarcoplasm *(S)*, collagen *(C)* and early phase of intercalated disc formation *(arrows)* (Six weeks, osmium tetroxide, uranyl acetate and lead stain, × 6300).



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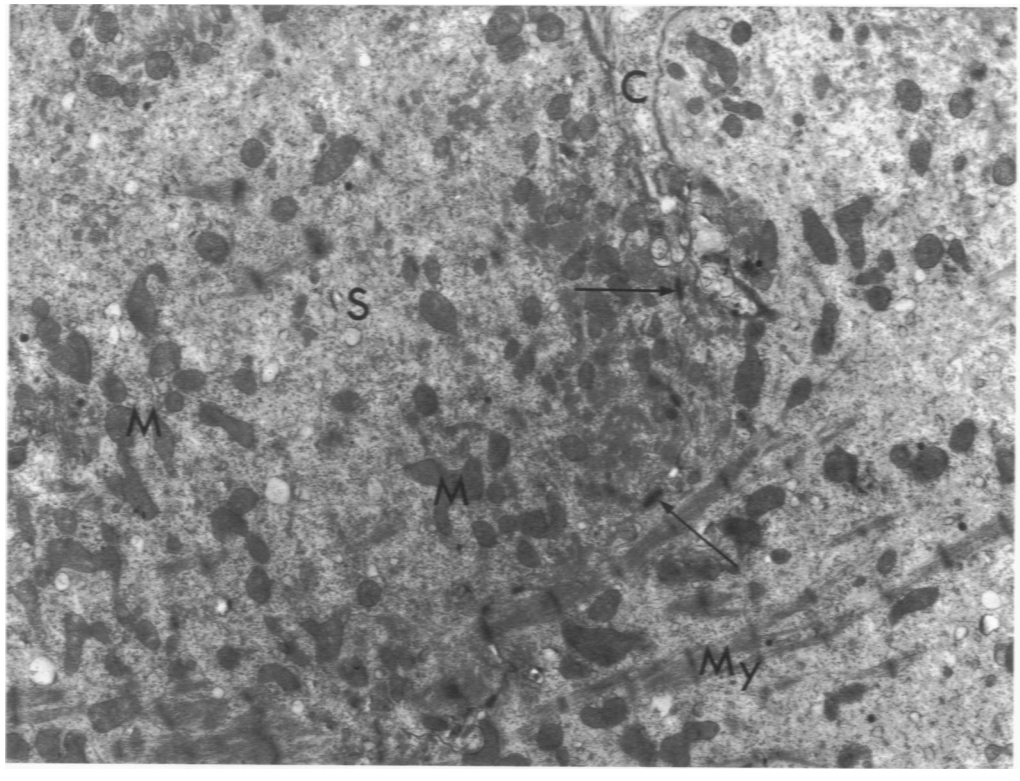

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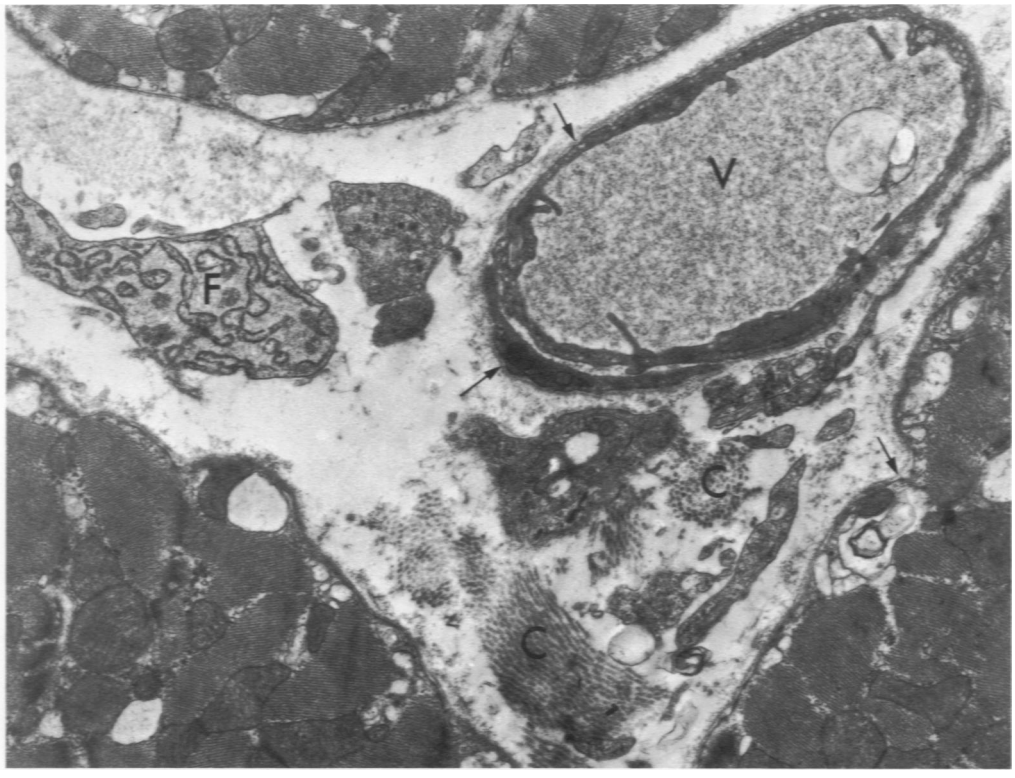

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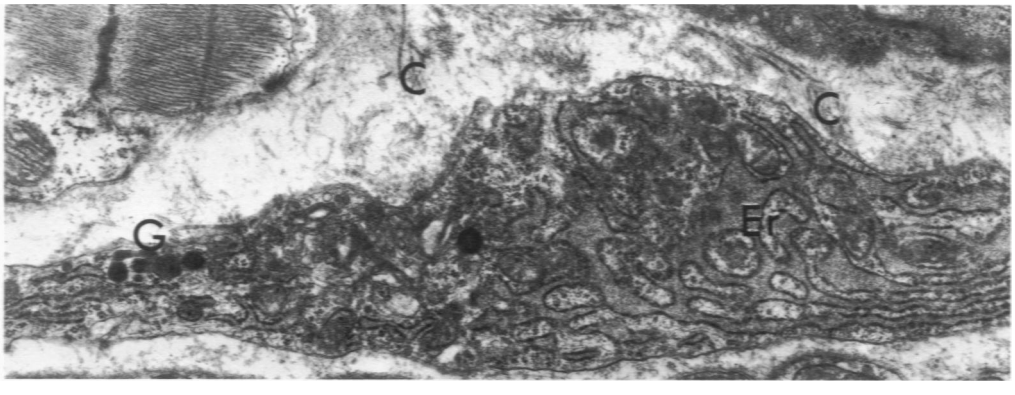

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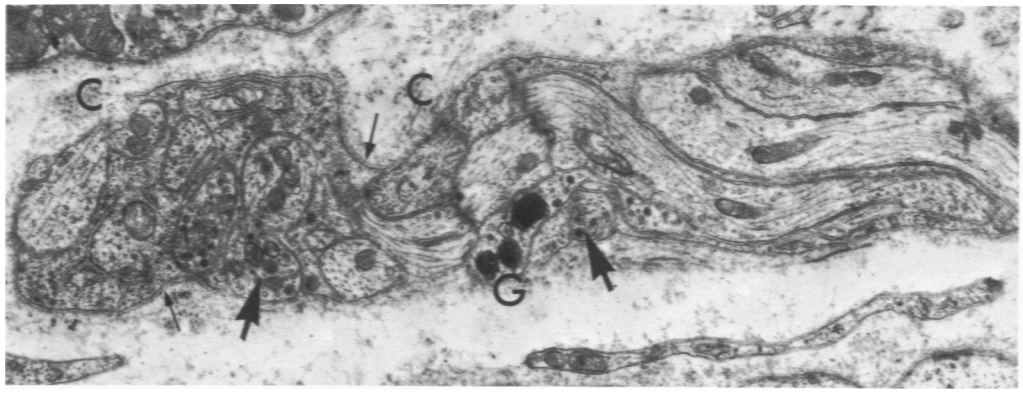
Fig 3—Electron micrograph of normal tissue showing a small vessel (*V*) fibroblast (*F*), collagen (*C*) and portions of three muscle cells. Also note basal lamina (*arrows*) (Six weeks, 3% isotonic glutaraldehyde and osmium tetroxide, uranyl acetate and lead stain, × 11,400). **Fig 4**—Electron micrograph of normal fibroblast showing the prominent ergastoplasmic cisternae (*Er*). Also evident are electron-dense granules (*G*) and extracellular collagen (*C*) (Six weeks, 3% isotonic glutaraldehyde and osmium tetroxide, uranyl acetate and lead stain, × 10,300).



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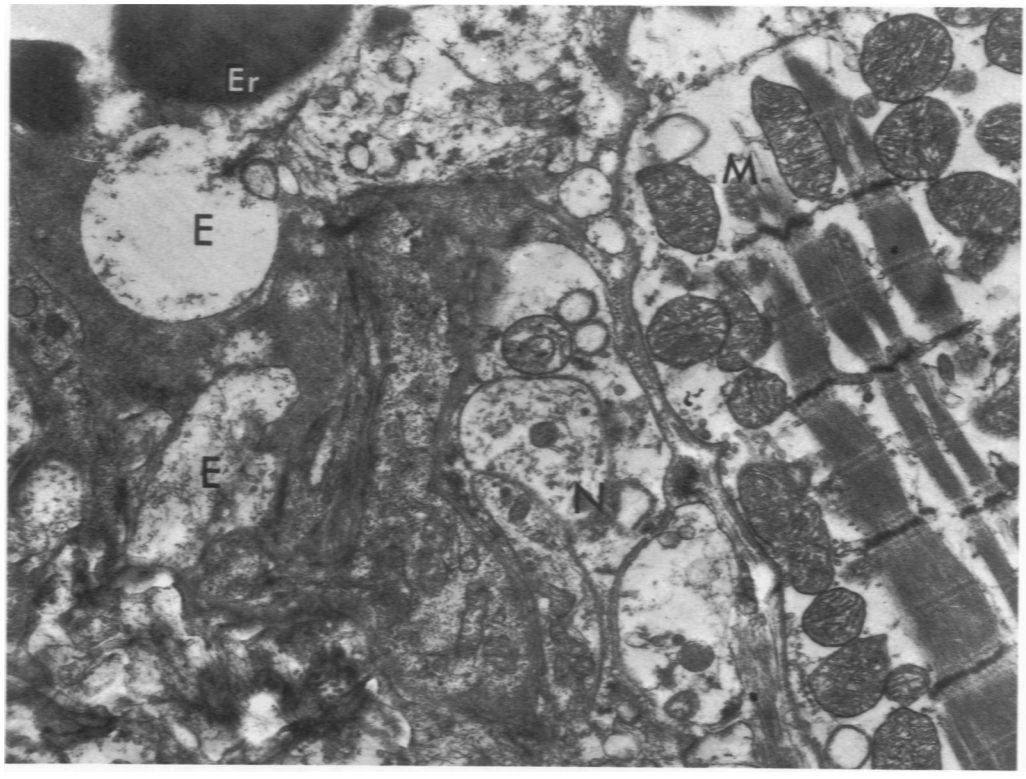

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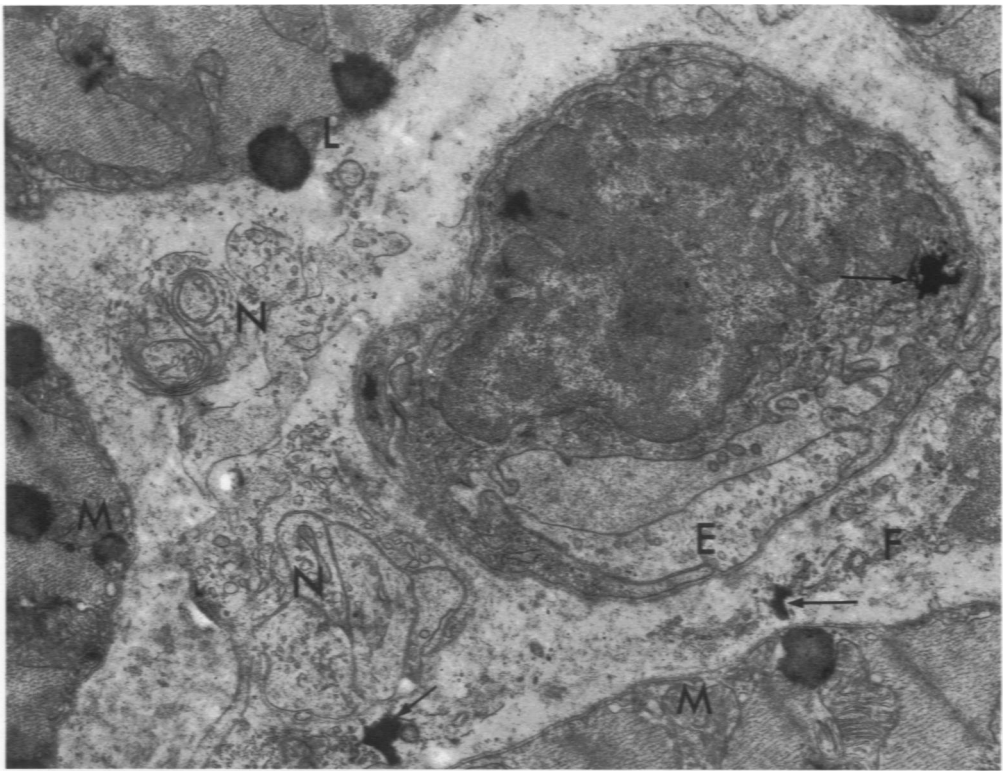
Fig 5—Electron micrograph of normal nonmyelinated nerve. Neurosecretory granules are evident (*large arrows*), as is collagen (*C*) and some electron-dense granules are also present (*G*). Note the thin basal lamina (*small arrows*) (Eight weeks, 3% isotonic glutaraldehyde and osmium tetroxide, uranyl acetate and lead stain, × 11,400). **Fig 6**—Electron micrograph of cardiac tissue showing gross alterations in cytologic elements. The muscle cells are degenerate (*M*). Also present are remnants of nerves (*N*), and capillary endothelium (*E*) with an erythrocyte (*Er*). Other unidentifiable elements are present. (Eight weeks, osmium tetroxide, uranyl acetate and lead stain, × 11,400).



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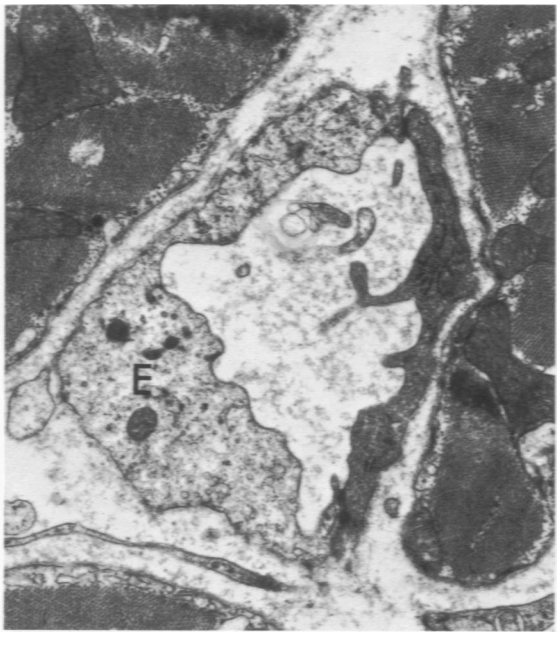

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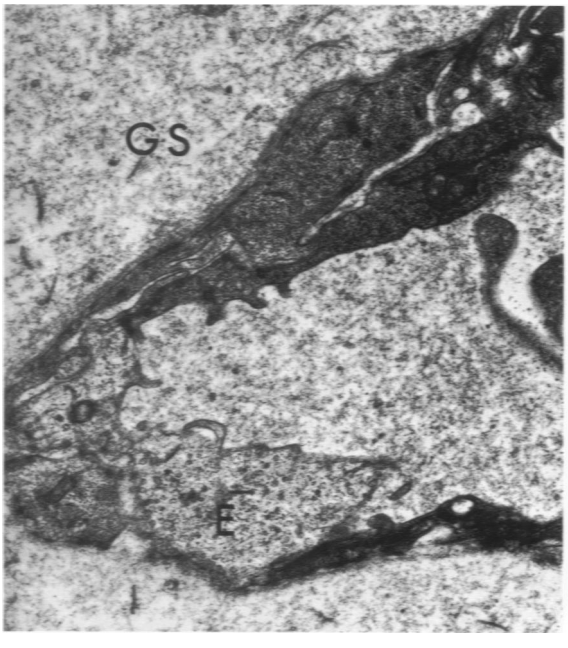

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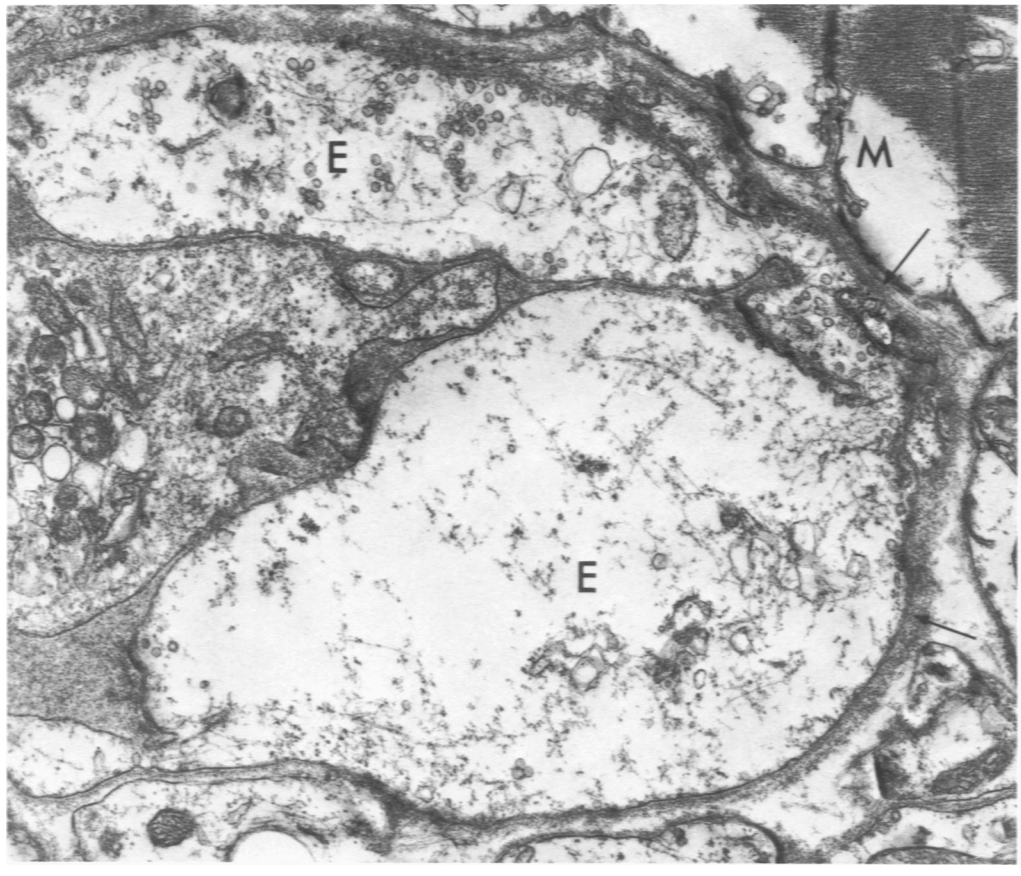
Fig 7—Electron micrograph of cardiac tissue from deficient piglet. Portions of three muscle fibers are present showing lipid droplets (*L*) and altered mitochondria (*M*). The capillary can be seen to contain an altered endothelial cell (*E*) and adjacent to this is a disrupted fibroblast (*F*) and nerve (*N*). All three elements contain electron-dense, lipid-like inclusions (*arrows*) (Six weeks, osmium tetroxide, uranyl acetate and lead stain, × 11,400). **Fig 8**—Electron micrograph of capillary showing degenerate endothelial cell (*E*). Portions of muscle cells are also present (Three weeks, 3% isotonic gutaraldehyde and osmium tetroxide, uranyl acetate and lead stain, × 12,400). **Fig 9**—Electron micrograph showing altered endothelial cell (*E*). Also note lack of extracellular collagen but prominent granular ground substance (*GS*) (Six weeks, 3% isotonic glutaraldehyde and osmium tetroxide, uranyl acetate and lead stain, × 11,400).



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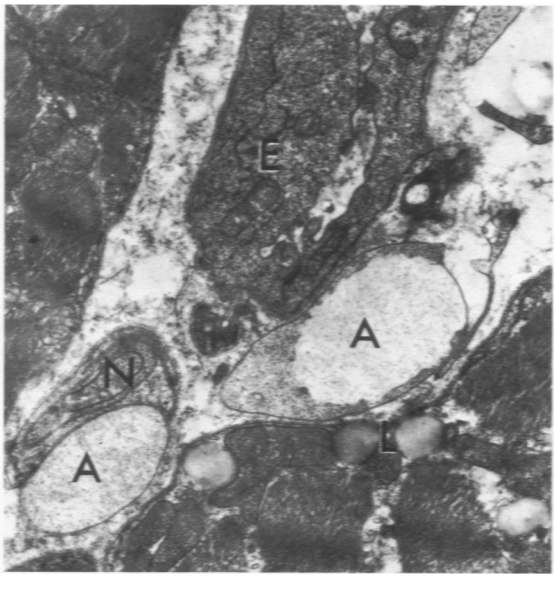

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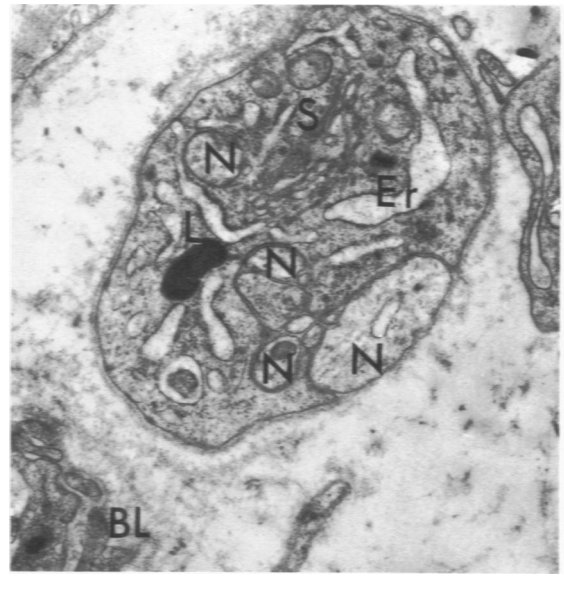

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Fig 10—Electron micrograph showing major alterations of the endothelium at this stage (*E*). Also note prominent basal lamina (*arrows*), and adjacent abnormal muscle (*M*) (Six weeks, osmium tetroxide, uranyl acetate and lead stain, × 16,800). **Fig 11**—Electron micrograph of degenerate nerve. A portion of a neuron can be seen (*N*); swollen axon portions (*A*) devoid of structure. The only observable change in the muscle is the presence of lipid droplets (*L*); note the lack of extracellular collagen. Also present is a capillary with altered endothelium (*E*) (Three weeks, 3% isotonic glutaraldehyde and osmium tetroxide, uranyl acetate and lead stain, × 8,800). **Fig 12**—Electron micrograph showing a portion of a Schwann cell (*S*) enclosing four nerves (*N*). The former shows dilated ergastoplasmic profiles (*Er*) and a lipid droplet (*L*). Note the lack of definite collagenous fibrils and the basal lamina (*BL*) of nearby capillary (Three weeks, osmium tetroxide, uranyl acetate and lead stain, × 9,300).

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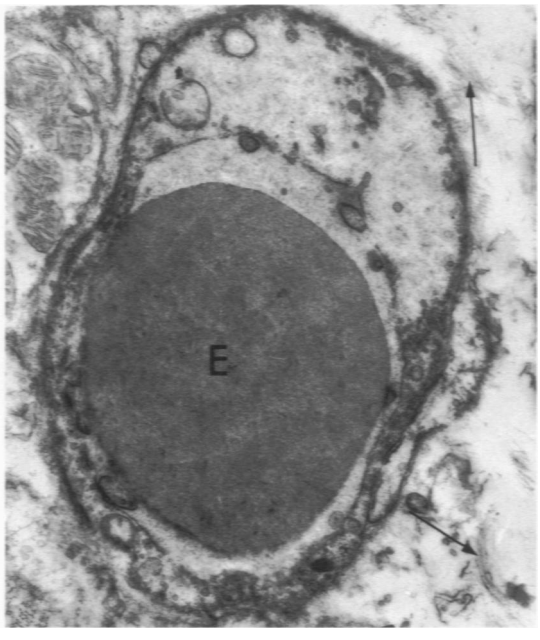

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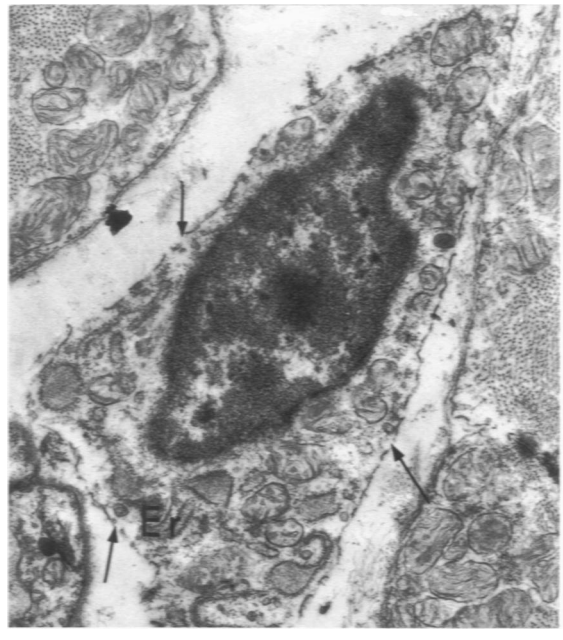

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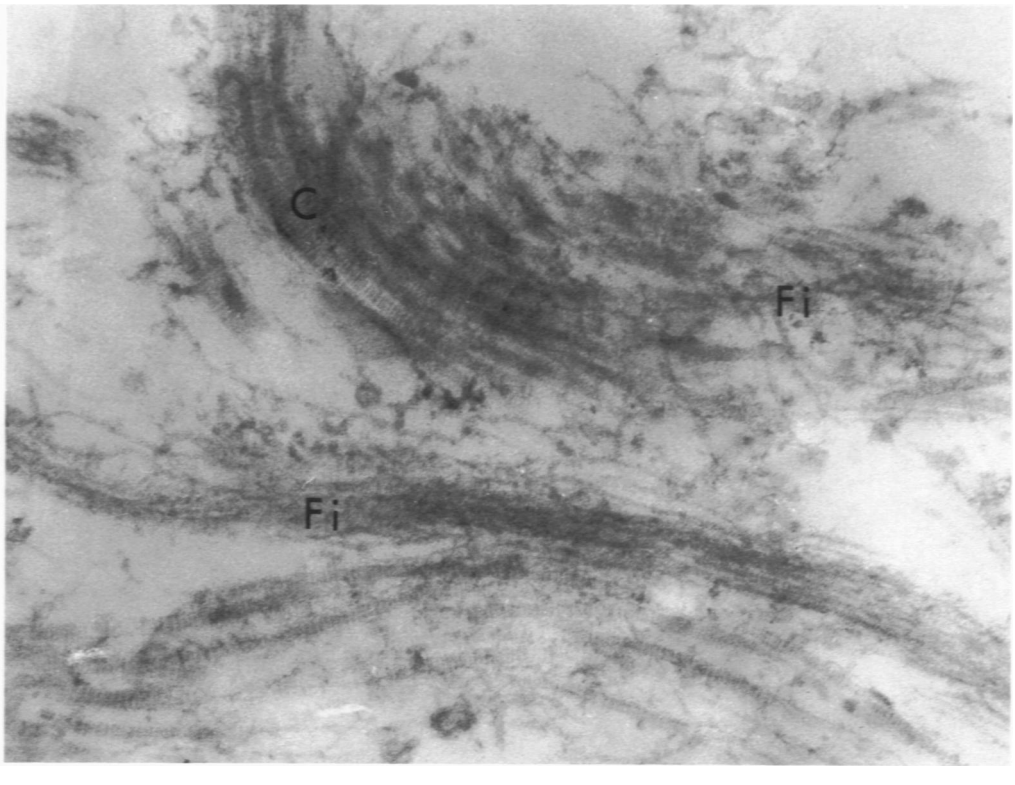

Fig 13—Electron micrograph showing a small degenerate capillary with enclosed erythrocyte (*E*) and fine extracellular filaments (*arrows*) (Newborn, osmium tetroxide, uranyl acetate and lead stain, × 13,800). **Fig 14**—Electron micrograph showing a degenerate fibroblast containing disorganized ergastoplasm (*Er*) and discontinuous plasma membrane (*arrows*). Also note the fine extracellular filaments (Newborn, osmium tetroxide, uranyl acetate and lead stain, × 15,600). **Fig 15**—Electron micrograph showing collagen fibrils (*C*) with an axial periodicity and associated filaments (*Fi*) (Newborn, osmium tetroxide, uranyl acetate and lead stain, × 70,300).